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Ecophysiological and metabolic response patterns to drought under controlled condition in open-pollinated maternal families from a *Fagus sylvatica* L. population

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ABSTRACT

The genetic variability within a plant species or population determines the extent to which drought impacts plant function at different levels of biological organization. By disentangling the extent to which drought tolerance is genetically determined within a population, we can get insights of the adaptive capacity of species to cope with climate change at the local scale. We compared the ecophysiological and leaf metabolic responses of different maternal families from a single population of the water-stress-sensitive species, *Fagus sylvatica* L, to an imposed drought in a controlled experiment. Nitrogen metabolism was modified by water stress, and some proteinogenic amino acids such as: proline (PRO), valine (VAL) and aspartic acid (ASP), as well as the non-proteinogenic amino-acid γ-aminobutyric (GABA), accumulated in response to drought. Gas exchange was depressed with increasing water deficit (i.e. a decrease in predawn water potential – Ψ_{pd}) in all families, with significant differences among maternal families for net photosynthesis (A_{net}) and stomatal conductance of water vapour (g_{wv}). However increasing stomatal limitation of A_{net} did not reduce the pool of non-structural carbohydrates (NSC). In fact, under water stress NSCs, including both mono- and disaccharides, accumulated as leaf Ψ_{pd} decreased; with the most striking effect of water stress being an increase in the trisaccharide raffinose (RAF) in all the maternal families. Leaf secondary metabolites were also affected differently by water stress among the maternal families. Overall, there were differential changes among our maternal families in various leaf metabolites, in seedlings under both the well-watered and water-stressed treatments. This result supports the hypothesis that there is a high degree of intra-population variability in the response to drought at different levels of biological organization in beech, i.e. ranging from ecophysiological to metabolic responses. This intra-population genetic variability is fundamental for adaptation of the species to drier environments at its trailing range edge in a context of climate change.

1. Introduction

Water scarcity and climate warming are considered the two most important environmental and selective pressures that constrain *Fagus sylvatica* throughout its geographical range (Leuzinger et al., 2005). This limitation is expected to become increasingly relevant, not only at the trailing distribution edge of beech in the southernmost range (Jump et al., 2006; Chen et al., 2015), but across its full range (Geßler et al., 2007; Cavin and Jump, 2017). Although beech tends to behave as an iso-hydric species, being very sensitive to drought in comparison with other coexisting forest tree species (Backes and Leuschner, 2000; Aranda et al., 2005; Bréda et al., 2006; Cano et al., 2013), it had relatively high resilience and capacity to recover from moderate water stress (Gallé and Feller, 2007; Sánchez-Gómez et al., 2013; Blessing et al., 2016). In addition, intra-specific variability in response to drought of phenological, morphological and functional traits is common among provenances of beech (Peuke et al., 2002; Rose et al., 2009; Robson et al., 2012, 2013; Psidova et al., 2015; Aranda et al.,

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2015), and likewise at the intra-population level when comparing maternal families (Aranda et al., 2017a). The functional response of species to drought at different ontogenic stages (i.e. from seedlings to mature trees) has been widely tested in recent years in various eco-physiological studies. Yet, there is scant information in this regard at other biological levels of organization (i.e. the impact of water deficit on the molecular profile of leaf metabolism). Previous molecular studies of beech assessed the role of antioxidant systems in response to light (Peltzer and Polle, 2001), the season (Polle and Morawe, 1995), or atmospheric pollutants such as ozone (Luwe, 1996). These studies focused on a limited pool of compounds or enzymatic systems that are highly responsive to the accumulation of reactive oxygenic species (ROS). Moreover, the metabolic response of beech to drought has been assessed through the study of leaf biochemical changes in important amino acids such as proline which is well known to respond to drought (Schraml and Rennenberg, 2000), or more recently through the analysis of the impact of water stress on the pool of leaf carbohydrates (Liu et al., 2017). Seasonal and daily patterns of carbohydrates, together with their inter-organ redistribution, have also been analyzed in depth (Hoch et al., 2003; El Zein et al., 2011). Furthermore, C^{13} labelling has been used to study carbon assimilation and mobilization between different sources-sinks in plants (Desalme et al., 2017). However, as far as we know, there are few precedents that have considered the whole range of metabolites involved in plant response to stress, and compare the role played by genetic background within the species in modulating these responses (but see Peuke et al., 2002 or Aranda et al., 2017b).

Biotic and abiotic environmental stresses are the main factors contributing to changes in the metabolome (Warren et al., 2011; Rivas-Ubach et al., 2014; de Miguel et al., 2016). The synthesis and accumulation of primary and secondary metabolites changes greatly according to the effective water stress experienced by a plant (Fernández de Simón et al., 2017). For primary metabolism, considered through the pool of non-structural carbohydrates (NSC), both mono- and disaccharides are frequently upregulated during the early phases of drought (Chaves, 1991), though the extent of this change can be highly species- and time-dependent (Rodríguez-Calcerrada et al., 2017). Further changes in polyols (e.g. *myo*-inositol) or the trisaccharide raffinose are related to the pool of NSC. Some of these carbohydrates (e.g. sucrose) or polyols (e.g. pinitol, quercitol, ribitol) are thought to be compatible osmolites. Beyond changes in primary metabolites related to carbon metabolism, nitrogen metabolism in beech is expected to respond to water stress (Fotelli et al., 2002; Rennenberg et al., 2009; Leberecht et al., 2016). Some amino acids, such as proline or the non-proteinogenic amino-acid GABA (γ-aminobutyric acid), acting as osmolites and/or involved in stress-signaling processes, are commonly reported to accumulate in various plant tissues in response to water stress (Bown and Shelp, 2016). Other amino-acids, such as serine or glycine − related to photorespiration, are highly sensitive to water limitation. These changes in primary metabolism would be expected to enhance drought tolerance (Merewitz et al., 2012).

Changes to leaf biochemical profiles of primary metabolites are usually associated with changes in many secondary compounds as well. Plant secondary metabolism mediates numerous ecological processes such as plant-herbivores interactions (O'Reilly-Wapstra et al., 2010; Rivas-Ubach et al., 2014), and plant response to environmental stressors such as drought (Gleadow and Woodrow, 2002; King et al., 2004; Rivas-Ubach et al., 2016) or warming temperatures (Wahid et al., 2007). The modification of primary and secondary metabolism in response to water stress, runs in parallel with changes in leaf functional processes related to carbon assimilation and regulation of water loss. The interplay between changes in functional response at different scales, from leaf physiological and morphological traits to metabolic responses, is only now beginning to be explored in forest tree species (Warren et al., 2011; Turner et al., 2016). Studying changes in the metabolic response of plants to environmental stress provides new insights into the complex interplay of the phenotypic changes between the molecular and the organ-or-individual level (Warren et al., 2011). However, only now we are starting to be able to harmonize the different approaches that address the complex responses of plants to the environment, allowing us to integrate plant responses across different biological levels of organization. Consequently, we can now study together both those functional processes, related to carbon fixation and control of water loss, and the modulation of the primary and secondary metabolism in crops (Turner et al., 2016) or forest tree species (Warren et al., 2011; Barchet et al., 2014; Granda et al., 2014) with which they are fundamentally linked.

It is well established that water stress brings about early stomatal closure in beech, and a decrease in net photosynthesis and quantum yield of PSII among other functional processes (Tschaplinski and Tuskan, 1994; Robinson et al., 2007; Robson et al., 2012; Sánchez-Gómez et al., 2013; Cano et al., 2013). These changes in the leaf physiology should be accompanied by modifications at the molecular scale with regulation of carbon and nitrogen metabolism. Thus, this study was intended to connect our current knowledge on the response to water stress at the ecophysiological level in beech, with the less well-described metabolic responses. Because of the relevance of intra-specific variability in beech for multiple phenotypic traits (Robson et al., 2013; Aranda et al., 2015), we considered several different open-pollinated families coming from the same population in our experiment. The main objectives of the study were: a) to evaluate the leaf metabolic response to water stress of a large pool of primary and secondary metabolites; b) to test whether differences among open-pollinated families would be reflected in the leaf metabolic response as previously reported for functional traits, and c) to examine the potential interlink between the most responsive of the metabolites and the ecophysiological response to drought.

2. Materials and methods

2.1. Plant material and experimental set-up

Beechnuts were harvested from branches of 11 mature trees in "Montejo de la Sierra" beech-wood (41°7′N, 3°30′W, Madrid, central Spain) just before seed fall in autumn. The stand is one of the southernmost examples of *Fagus sylvatica* L. at the trailing edge of its distribution range in the Iberian Peninsula, and is subject to frequent dry periods (Aranda et al., 2000, 2005; Cano et al., 2013; Dorado-Liñán et al., 2017). The seeds were maintained at 4 °C for two months, and afterwards were chilled for 8–10 weeks in individual trays. Germinated seeds were careful planted during the middle of April in a greenhouse in containers of 400 cm^3 filled with a 3:1 volume mixture of peat Floragard TKS2 (Floragard Vertriebs gmbh, Oldenburg, Germany) and washed river sand. The substrate was supplemented with 2 $kg\,m^{-3}$ of Osmocote Plus balanced fertilizer (16-9-12 NPK + 2 micronutrients; Scotts, Heerlen, The Netherlands). The experimental was designed to evaluate the effects of two main factors: open-pollinated family, hereafter maternal families, and water availability. After germination and seedlings establishment under well-watered conditions, twenty seedlings per maternal family were split into two groups: well-watered (WW) and water-stressed (WS), and arranged randomly within each of two blocks in the greenhouse to control for potential environmental gradients within the greenhouse. All plants were watered regularly until the first true leaves were fully expanded in early June and had fully matured. Afterwards, WS seedlings were submitted to weekly cycles of water-stress by deprivation of watering for one week, followed by full rehydration of pot substrate to full water-holding capacity after the 7 days of watering deprivation. This protocol was followed over 11 weeks. Environmental conditions in the greenhouse were: 500–600 $\mu mol\,m^{-2}\,s^{-1}$ of PPFD, 65 ± 5% of relative humidity, maintained with a misting system and maximum daily temperature not exceeding 28 °C. For more details of the experimental design, see Aranda et al. (2017a).

2.2. Measurement of ecophysiological traits

Ecophysiological data represent a subsample from a previous ecophysiological study with a higher pool of families (see Aranda et al., 2017a for more details). In brief, and after 11 weekly cycles of water-stress/rehydration, the predawn leaf water potential (ψ_{pd}) was measured in all seedlings with a Scholander pressure chamber (PMS Instrument Co. 7000, Corvallis, OR. USA). The VWCs was also recorded in the pot substrate of all seedlings just after measuring ψ_{pd}. The night before measuring water status, all seedlings were brought to a climate chamber (Fitoclima 10000 EHHF, Aralab, Portugal). The photoperiod in the chamber was set to match the natural photoperiod experienced by the plants in the greenhouse during growth. Climate conditions in the growth chamber during measurements were: temperature of 20 °C and 70% relative humidity/night and 25 °C–65% relative humidity/day. One hour after beginning of the daily light period, different gas exchange and chlorophyll fluorescence parameters were recorded: net photosynthesis on a leaf area basis (A_{area}), stomatal conductance of water vapour (g_{wv}) and leaf chlorophyll fluorescence with a portable photosynthesis system (LiCor 6400 XP, Li-COR Inc., Lincoln, NE, USA) coupled to an integrated fluorescence chamber (chamber Li-6400–40, Li-COR Inc., Lincoln, NE, USA). Gas exchange was measured at a saturating PPFD of 1200 μmol m^{-2} s^{-1}, enough to achieve maximum values of A_{sat}, VPD around 1.2KPa, air temperature close to 25 °C and a constant CO_2 in the inflow air of 400 ppm. Actual photochemical efficiency of photosystem II (φ_{PSII}) was estimated according to Genty et al. (1989): $\Phi_{PSII} = (F_{m'} - F_s)/F_{m'}$, after measuring steady-state fluorescence (F_s) and maximum fluorescence during a saturating light pulse ($F_{m'}$).

2.3. Leaf metabolic profiling

At the end of the drought period, leaf metabolic profiling was carried out on each plant using freeze-dried leaves harvested at dawn, run using two independent approaches: untargeted GC–MS (Gas Chromatography-Mass Spectrometry) analysis, and targeted LC-DAD (Liquid Chromatography-Diode Array Detector) analysis of polyphenolic compounds. Extraction and instrument analysis of metabolites were carried out following the method described in Cadahía et al. (2015). Samples (5 mg) were first extracted with chloroform (200 μL) into an ultrasonication bath for 15 min at room temperature, thereafter water/methanol (1/3 v/v, 800 μL) containing the internal standard (methyl-β-D-galactopyranoside) was added to the mixture, applying ultrasonication again for 20 min. The extracts were centrifuged; 200 μL of supernatant, after being dried under a nitrogen stream, were used for GC–MS analysis and the remaining supernatant directly analyzed by LC-DAD. The samples destined for GC–MS analysis were derivatized using methoxyamine hydrochloride in pyridine, and *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA).

GC–MS analysis was performed using an Agilent 6890N GC system gas chromatograph (Palo Alto, CA) equipped with a quadrupole mass spectrometer Agilent 5973N, and a HP-5 capillary column (30 m × 0.25 mm i. d., film thickness of 0.25 μm, Agilent, Madrid, Spain) with helium as the carrier gas at a constant flow rate of 1.1 mL min^{-1}. The oven temperature was 70 °C, and increased at 3 °C min^{-1} to 300 °C, and then held at this temperature for 15 min; while the injection temperature was 270 °C. Detection was performed by electron impact mode with ionization energy of 70 eV. Finally, the interface detection temperature was 290 °C (MS source at 230 °C, and MS quadrupole at 180 °C). The chromatographic peaks were identified using a private database generated from more of 240 pure commercial standards, and commercial libraries: Agilent Fiehn GC–MS Metabolomics RTL Library, and Wiley7/Nist05GC/MS Library. Quantitative valuation was carried out by applying the internal standard method, using peak areas from selected masses. The concentrations of each compound were obtained by comparison with calibrations made with pure reference compounds analyzed under the same conditions, and by calibration against a similar compound when the pure reference standard was not available or for unidentified compounds. Thus, methyl-phosphate and 3,4-dihydroxydihydrofuranone were quantified with phosphate and d-arabino-1,4-lactone calibration respectively, unidentified monosaccharides with d-glucose, sugar acids with threonic acid, sugar acid γ-lactones with xylonic acid γ-lactone, disaccharides with sucrose, sugar alcohols and *myo*-inositol phosphate with *myo*-inositol, glyceryl glycosides with glycerol, neophytadiene with phytol, ascorbic acid with dehydroascorbic acid, caffeoylthreonic acid isomers with caffeic acid, and caffeoylquinic acid isomers with chlorogenic acid.

LC-DAD analysis of polyphenolic compounds was performed using an Agilent 1100 L liquid chromatography system equipped with a diode array detector (DAD), and managed by a Chemstation for LC 3D systems Rev B.03.02 (Agilent Technologies, Palo Alto, CA, USA). The column was a 200-mm- × -4-mm i.d., 5 μm, Hypersil ODS C18, maintained at 30 °C and protected with a 4-mm- × -4 mm i.d. guard column of the same material (Agilent Technologies). The LC profiles were monitored at 325 ± 150 nm, and the UV/Vis spectra were recorded from 190 to 400 nm. The volume injected was 40 μL. The elution conditions were as follows: flow rate, 1 mL min^{-1}; temperature, 30 °C; solvent A = water/phosphoric acid (999/1, v/v), solvent B = methanol/phosphoric acid (999/1, v/v); gradient profile, 0–20 min, 100–85% A, 20–30 min, 85–75% A, 30–50 min, 75–50% A, 50–70 min 50–0% A, 70–75 min 0% A. Detection was performed by scanning from 190 to 400 nm. Peak identification was based on previous LC-DAD-MS (Liquid Chromatography-Diode Array Detector-Mass Spectrometry data) (Cadahía et al., 2015). The quantification of compounds was carried out by the external standard method. The concentration of each substance was measured by comparing it with a calibration made with the pure standard compound analyzed under the same conditions. Calibration of a structurally-similar compound was used when the pure reference standard was not available. Thus, caffeoylquinic acid isomers were quantified with chlorogenic acid, caffeoylthreonic acid isomers with caffeic acid, p-coumaroylthreonic acid with p-coumaric acid, feruloylthreonic acid with ferulic acid, and flavonol glycosides and other derivatives with their respective aglycones, quercetin and kaempferol.

2.4. Statistics

An univariate approach was used to test for the effect of water stress and maternal family on each ecophysiological variable and metabolite in a linear mixed effects model (LMM). Restricted maximum likelihood estimation and lmer function in the R package lme4 were used in the LMM (Bates et al., 2015). The watering treatment was a fixed factor (α), as was the maternal family (β) while the block (γ) was considered as a random factor according to the model in the general form:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha_i \times \beta_j) + \varepsilon_{ijk} \quad (1)$$

and where Y_{ijk} is the phenotypic value for each functional trait and metabolite in the k_{th} block for the j_{th} family of the i_{th} treatment, μ the mean, α the watering effect, β the maternal family effect, γ the random block effect, $(\alpha \times \beta)$ the effect of the interaction of treatment and family, and ε_{ijk} the residual variance. Only the interaction of family vs. watering treatment was included in the model, as the interaction of block was not significant with either watering or family. Significance of the different factors was determined from ANOVA (anova function in R), by comparing sequential and hierarchical models where one of the factors was drawn and compared with the general model considering only the block as random factor. Water stress was highly significant for most metabolites and ecophysiological traits (see below), thus supervised multivariate data analysis was carried out to establish the impact of water stress in the pool of data as a whole. An orthogonal projection to latent structured discriminant analysis (OPLS-DA) was

used to make inferences about those metabolites with highest influential weighting in response to water stress (Bylesjö et al., 2006; Wiklund et al., 2008). The final model included the watering class summarizing the correlated variation in the predictive component (t_p), and two more orthogonal components (1 + 2) containing the uncorrelated variation that was not related to the separation of the two watering treatments (t_o). Response variables were log transformed to meet the assumptions of normality and homogeneity of variance for the univariate analysis, and were pareto-scaled before multivariate analysis. We used S plots to discriminate those metabolites and functional traits with highest loading in the response to water stress.

A heat-map from the z-transformed median values for each metabolite in seedlings from both the water-stressed and well-watered treatments was constructed to estimate similarity of the leaf metabolic response among the different families. Only metabolites that showed a significant variation according to family in the univariate analysis were included in the heat-maps.

To consider the relationship between ecophysiological traits and the metabolic profiles, a partial least-squared regression model (PLS) was constructed for those functional traits related to carbon assimilation, and considered as Y-dependent variables in the model: leaf net photosynthesis (A_{net}), stomatal conductance of water vapour (g_{wv}) and quantum yield of PSII (Φ_{PSII}). Those metabolites that varied in response to water stress and/or maternal family were considered in the X matrix as dependent variables. Models with three components achieved the maximum Q^2 value for the three Y-dependent variables. Auto-scaling of all variables to unity was carried out before fitting the models. The projection in the model of each metabolite (VIP), and its PLS model coefficient, were considered as criteria in the selection of the most important variables. Those metabolites with a VIP > 1, and PLS-regression coefficients (mean ± 1 SE) different from 0 were considered as relevant in the final models. The confident intervals for PLS-regression coefficients of each variable were obtained by jack-knifing (Efron and Gong, 1983). The cumulative Q^2 (Q^2cum) and R^2 (R^2Ycum) of each model were considered as criteria for judging the quality of the models, followed by CV-ANOVA to check the reliability of the models (Eriksson et al., 2008), and were used to assess readability of the fitted models. All the multivariate analyses were carried out with the software SIMCA-P+ (SIMCA-13.0, Umea, Sweden, Umetrics, Inc.).

3. Results

3.1. Overall drought response

The water stress treatment that we applied brought about a decrease in the Ψ_{pd} of the plants of about 1 MPa, from $\Psi_{pd} = -0.25 \pm 0.02$ MPa in WW plants to $\Psi_{pd} = -1.25 \pm 0.02$ MPa in WS plants. The largest changes in ecophysiological traits were in response to water stress, although a smaller effect of family was also present for A_{net} and g_{wv} (Fig. 1). Changes in Φ_{PSII} were mostly explained by the watering treatment, without significant differences among families within each watering treatment.

Among the 110 metabolites distinguished from GC–MS and LC-DAD analyzes, 86 were positively identified, 19 tentatively assigned a chemical structure, and the rest remained as unidentified compounds. Mixed models for the effects on each metabolite revealed that drought was the main factor that affected the content of many of the leaf metabolites in the leaves of the beech seedlings, with 69 compounds changing according to watering treatment (Supplementary material Table S1). Family also significantly affected the leaf concentration of some 31 compounds, without an interaction effect between treatment and family in most cases. Only eight metabolites: quinic acid, a sugar acid (SUG-2370), a disaccharide (DIS-2706), ribitol, and four glycosylated polyphenols (KMP-PEN, KMP-HC4-DO2, QUE-diHC4-DO and KMP-HC4-FER-DO), specifically differed in concentration depending on the maternal family, irrespective of watering treatment. Qualitative

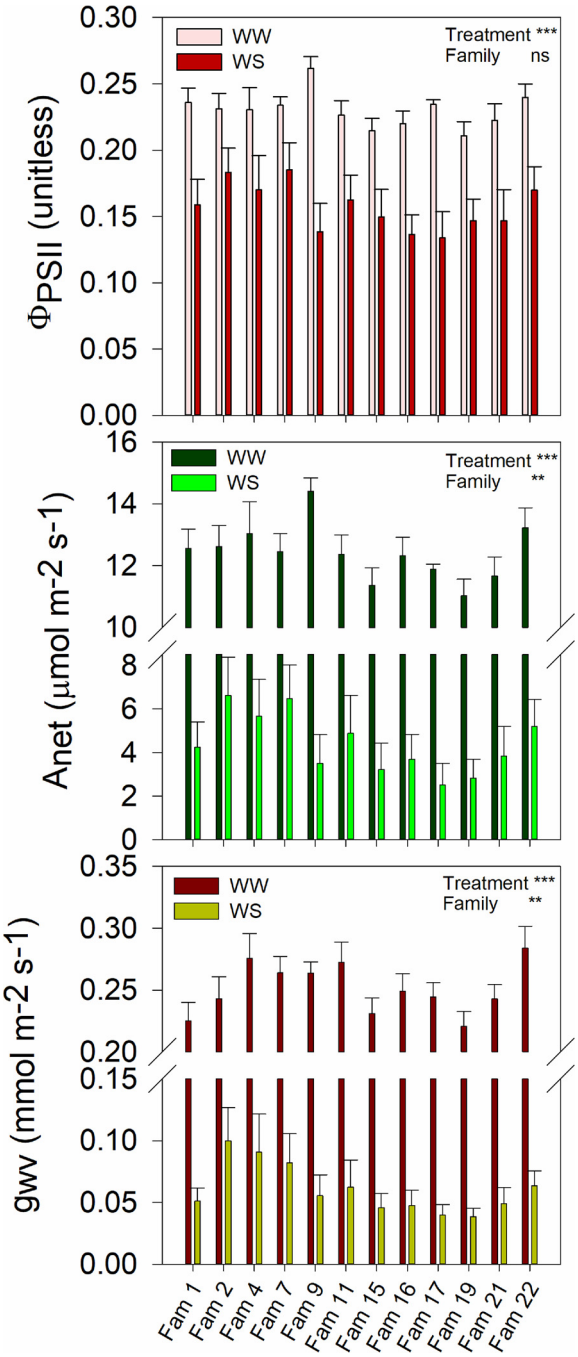

Fig. 1. Physiological variables at the peak of water deficit in leaves of beech seedlings from 11 maternal families, under well-watered (WW) and water-stressed conditions (WS). Values represent mean ± 1 SE, n = 7–10. ΦPSII (effective quantum yield of photosystem II); A_{net} (net photosynthesis on leaf area basis); g_{wv} (stomatal conductance of water vapour).

responses were similar among families for the rest of metabolites, and in some cases differences were apparent, though only the unidentified disaccharide DIS-2604 gave a significant maternal family × treatment interaction.

The multivariate OPLS-DA model contained one predictive and two orthogonal components (1 + 2) with a $R^2Y = 0.811$ as the sum of variation in the separate treatment (WW vs. WS), and $Q^2 = 0.763$ as confidence criterion showing the goodness of the model. Both indexes indicate that the model discriminated well between plants submitted to water stress and well-watered plants (Fig. 2). The total variation in X (the pool of ecophysiological traits and metabolites) that was correlated

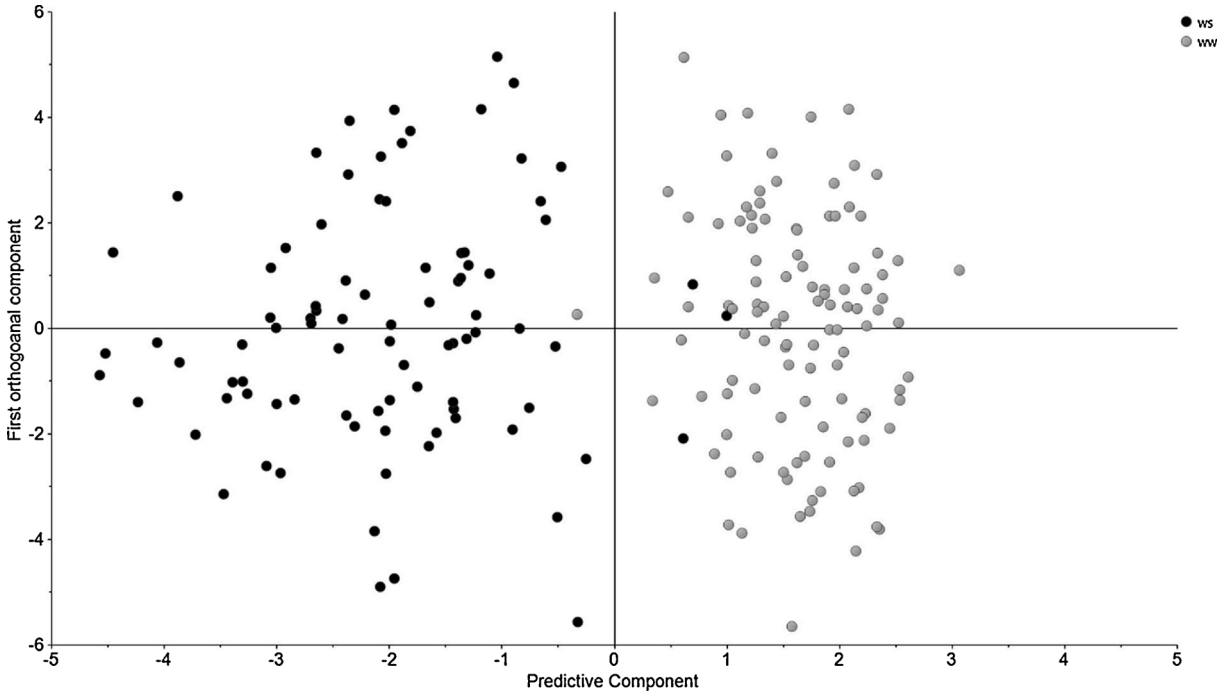

Fig. 2. An orthogonal projection of partial least square discriminant analysis (OPLS-DA) at the peak of water deficit in leaves of beech seedlings constructed from ecophysiological traits and metabolites. Black points represent well-watered plants, and grey dots plants that were water-stressed. Orthogonal components 1 and 2 explained 15.7% and 8.79% of the variation respectively, and the predictive variable explained 13.5% of the variation in the pool of physiological traits and metabolites (X).

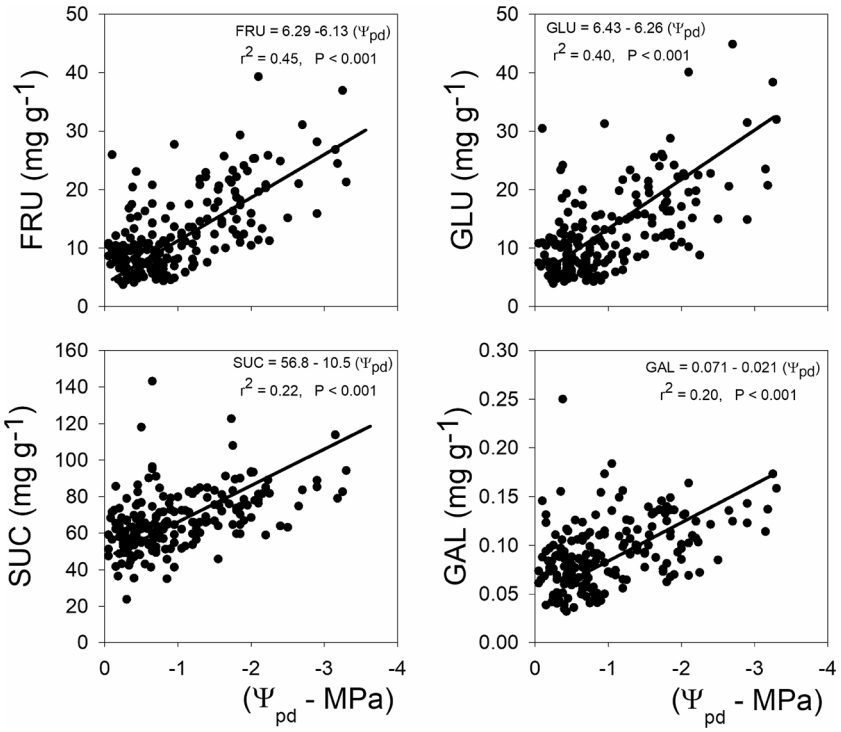
FRU = 6.29 -6.13 (Ψpd)
r2 = 0.45, P < 0.001
FRU (mg g-1)
GLU = 6.43 - 6.26 (Ψpd)
r2 = 0.40, P < 0.001
GLU (mg g-1)
SUC = 56.8 - 10.5 (Ψpd)
r2 = 0.22, P < 0.001
SUC (mg g-1)
GAL = 0.071 - 0.021 (Ψpd)
r2 = 0.20, P < 0.001
GAL (mg g-1)
(Ψpd - MPa)
(Ψpd - MPa)

Fig. 3. Relationship between predawn water potential as surrogate of the effective water stress endured by beech seedlings (Ψ_{pd} – MPa) and the four most abundant carbohydrates: FRU – fructose; GLU – glucose; SUC – sucrose; GAL – galactose. The linear models and significance are included in the graphs.

with Y (watering treatments) was 13.5%, and the un-correlated variation 24.5%. In this model, A_{net} and g_{wv} from the ecophysiological traits, and some metabolites related with primary metabolism were the variables with highest loading in the first predictive component according to the VIP value (Fig. S1). With increasing water deficit, there was a general trend towards lower values of A_{net}, g_{wv}, and Φ_{PSII} (consistent with the univariate analysis), but a general increase in the pool of monosaccharides and disaccharides. Those monosaccharides that accumulated most in response to drought were the hexoses: glucose, fructose and galactose, and the pentose ribose. The response of the main non-structural carbohydrates (NSC) to water stress was confirmed by the relationship between the seedling water status, assessed from the $\Psi_{pd,}$ and the NSC concentration in leaves (Fig. 3). For the disaccharides, sucrose levels significantly built-up between 1.1–1.5 fold on average in

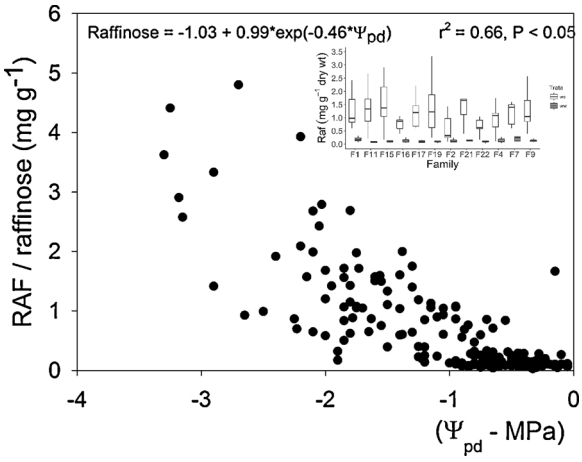
Raffinose = -1.03 + 0.99*exp(-0.46*Ψpd)
r2 = 0.66, P < 0.05
RAF / raffinose (mg g-1)
(Ψpd - MPa)
Raf (mg g-1 dry wt)
F1 F11 F15 F16 F17 F19 F2 F21 F22 F4 F7 F9
Family

Fig. 4. Relationship between predawn water potential as surrogate of the effective water stress endured by beech seedlings (Ψ_{pd} – MPa) and the raffinose. At the top, the non-linear model that best explained the observed trend is shown (equation, pseudo r^2 and significance of the model). Differences among maternal families are also depicted as a boxplots in the inset graph, and with well-watered plants depicted in white boxes and water-stressed plants in grey.

WS vs WW plants across families in general, without significant changes in maltose, or the other identified disaccharides. The concentrations of most of the remaining unidentified disaccharides increased significantly under water stress (inferred from the Ψ_{pd}) (Fig. S2). The oligosaccharide raffinose was the most responsive carbohydrate to water stress, its concentration increased between 3.2–14.7 folds in leaves of WW vs. WS plants across maternal families; an exponential increase in leaves with Ψ_{pd} under −1.0 MPa (Fig. 4). Raffinose was the metabolite with highest weighting in the OPLS-DA model, clearly discriminating between WW and WS plants (highest VIP, see the Supplementary Fig. S1). The reliability of the change due to WS of any of the pool of metabolites was highest for raffinose, followed by proline and γ-aminobutiric acid (GABA) (highest pcorr in the S-Plot, Fig. S1). The group of polyols, *myo*-inositol, *myo*-inositol-phosphate, *chiro*-inositol, and mannitol had higher concentrations in leaves of WS than WW seedlings, but ribitol and galactinol did not change significantly with watering treatment. Among compounds related to lipid metabolism, glycerol and glycerol-3-phosphate tended to increase with water stress. However, these changes were quantitatively of little relevance, between 0.93–1.62 fold for glycerol and 0.97–1.94 fold for glycerol-3-phosphate according to family. Less noticeable although still statistically significant was the slight decrease in lauric and palmitic acids with WS (0.86 and 0.91 fold respectively after averaging all families).

Among the carboxylic acids, three important acids from the TCA cycle increased in response to water stress: malic, fumaric and citric acids (Fig. 5). There were no changes in the α-ketoglutaric and succinic acids, which are also intermediates of the TCA cycle. However, there was a noticeable change in GABA which is connected to the TCA cycle through the GABA shunt, it increased between 1.6–7.9 fold with WS among families (Fig. 5). The amino acid, glutamic acid, a precursor of GABA, followed a similar pattern as GABA, increasing with WS: it was one of few amino acids that also changed according to maternal family (see below). The unidentified sugar acid SUG-1581 increased slightly with WS and consistently across the pool of maternal families. Together TCA compounds as a group, and other metabolites related to nitrogen metabolism were also significantly modified in response to drought. Thus, most amino acids responded by accumulating higher concentrations in the leaves of seedlings submitted to WS. In particular, the most noticeable changes were in proline (Fig. 6) and that already mentioned for GABA. Other amino acids that increased in response to WS were: valine, aspartic acid, isoleucine, threonine, serine and glycine (Fig. 6).

The response to drought of some secondary metabolites varied significantly. Thus, phenolic compound related to phenylpropanoid metabolism, such as the *p*-coumaric, ferulic, caffeic, chlorogenic and neochlorogenic acids, and together the flavanols, epicatechin and catechin, related to flavonoid biosynthesis, all decreased significantly in response to drought for the most of families (Fig. 7), although the shikimic and quinic acids did not significantly change (Table S1). The flavonol derivatives, on the whole also changed in response to drought, decreasing in general as result of the WS endured by plants. Only quercetin-*O*-glucuronide, quercetin-*O*-pentoside, quercetin-*O*-glucoside and quercetin-*O*-deoxyhexoside, together the derivatives of caffeic acid, caffeoyl-quinic acid, the two isomers of caffeoyl-threonic acid, and the derivatives of kaempferol, kaempferol-*O*-glucoside and kaempferol-*O*-dipcoumaroyl deoxyhexoside, decreased noticeably in response to WS in all maternal families (Fig. 7). There was also a large change in another two unidentified compounds (RI-1570 and RI-1741) in response to WS.

3.2. Differentiation among families in the metabolome

Differences in the pool of metabolites among families were restricted to a small number of metabolites compared with those changing in response to water stress. Although some metabolites did increase in a similar general pattern across most families in response to drought, the magnitude of this change tended to differ according to family (Fig. S3). Of those metabolites with high inter-family variation in response to water stress, raffinose is the most striking and varied from 5 to 7 fold according to family. Amino acids such as aspartic and glutamic acids, isoleucine or serine also displayed large differences in responsiveness to water stress among families: in some families such as 11 and 21 they hardly changed, but in others such as families 19 and 22 they increased almost 4 fold. Of those metabolites that decreased in response to WS, the polyphenols, caffeoyl threonic (isomer 2), chlorogenic and neochlorogenic acids, were those that showed most differences among families, although they were not so noticeable as those of some of the previous classes of metabolites listed, producing from no meaningful changes in concentration in families 4 and 11, but significant decreases near to 0.2 fold in families 2, 15, 16 and 19 for instance. In addition, there were significant constitutive differences in metabolite content among families when plants were WW, or when families were compared under WS treatment (Fig. S4). In this respect, inter-family differences in polyphenols derived from caffeic acid were noteworthy. The caffeoylquinic acid isomer 1 concentration was between 0.8 μg/mg (family 11) and 2.4 μg/mg (family 21), and the chlorogenic acid concentration was between 2.6 μg/mg (family 1) and 11.3 μg/mg (family 21), for WW plants. This relationship between families in variability was maintained in WS plants, with intervals between 0.5 μg/mg (family 1, 15) and 1.1 μg/mg (family 11) for caffeoylquinic acid, and between 0.7 μg/mg (family 15) and 3.6 μg/mg (family 11) for chlorogenic acid. However, in general clustering of families according to the pool of analyzed metabolites changed from WW to WS plants.

3.3. Relationship between carbon assimilation traits and leaf metabolites

Three PLS models were adjusted to establish the relationship between leaf ecophysiological traits (A_{net}, g_{wv} and Φ_{PSII}) and the leaf concentration of different metabolites. The PLS regression models for A_{net} and g_{wv} produced a moderately good fit ($R^2 > 0.7$) and predictive capacity ($Q^2 > 0.6$), whereas the model for Φ_{PSII} performed poorest, with a $Q^2 < 0.5$ (Table 1). The metabolites with the highest loads in PLS models were similar for A_{net} and g_{wv}. Both models comprised of compounds linked mainly to carbon metabolism with negative coefficients, and in particular several sugars, e.g. raffinose, fructose, glucose and galactose (Table 1). Other compounds e.g. ni-1570, or the amino acid proline and ascorbic acid, were also negatively related to A_{net} and g_{wv} (i.e. a decrease in both gas exchange traits agrees with an increase

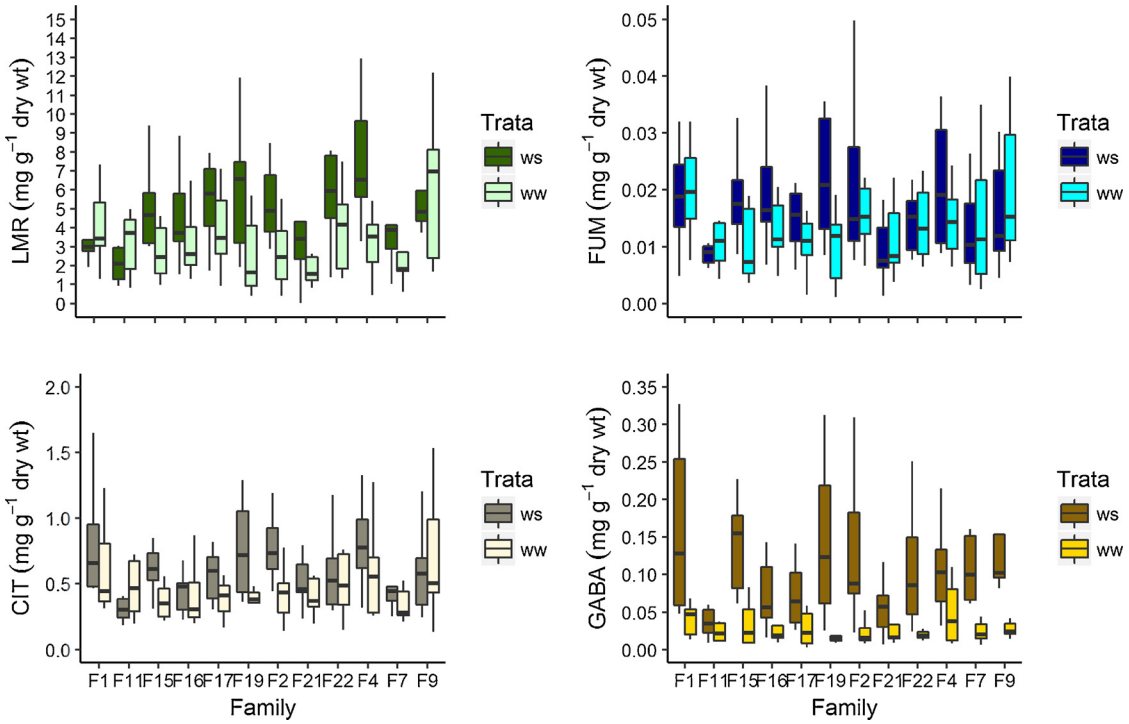

Fig. 5. Variation in leaf concentration of three organic acids from the TCA cycle and the related non- proteinogenic amino acid GABA, for well-watered (ww – bright colour) and water-stressed (ws – dark colour) seedlings in 11 maternal families of beech coming from the same beech population at the southernmost distribution limit of the species. LMR – malic acid; FUM – fumaric acid; CIT – citric acid; GABA – γ-amino-butyric acid.

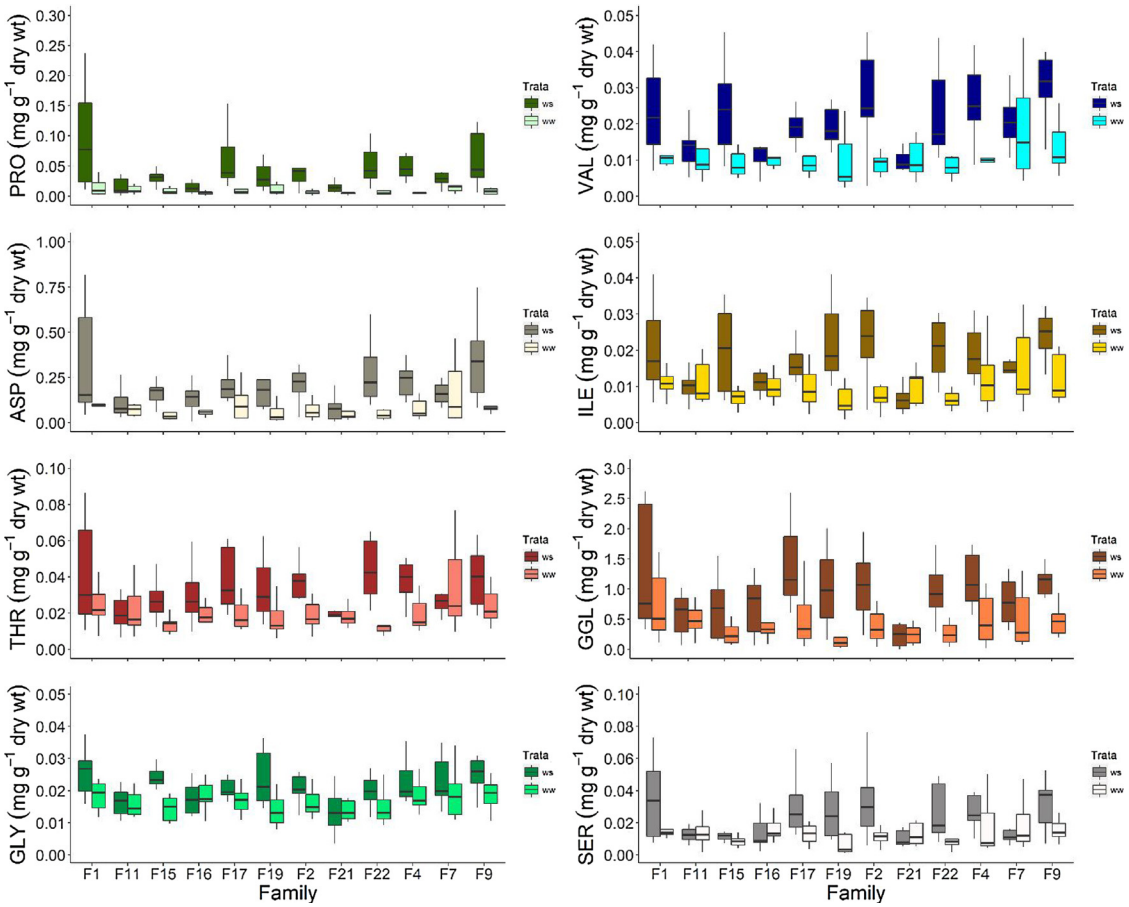
PRO (mg g−1 dry wt)
VAL (mg g−1 dry wt)
ASP (mg g−1 dry wt)
ILE (mg g−1 dry wt)
THR (mg g−1 dry wt)
GGL (mg g−1 dry wt)
GLY (mg g−1 dry wt)
SER (mg g−1 dry wt)
Trata
ws
ww
F1 F11 F15 F16 F17 F19 F2 F21 F22 F4 F7 F9
Family

Fig. 6. Variation in leaf concentration of 8 amino acids in well-watered (ww – bright colors) and water-stressed (ws – dark colors) seedlings of 11 maternal families coming from the same beech population at the southernmost distribution limit of the species. PRO – proline; VAL – valine; ASP – aspartic acid; ILE – isoleucine; THR-threonine; GGL – glutamic acid; GLY – glycine; SER – serine.

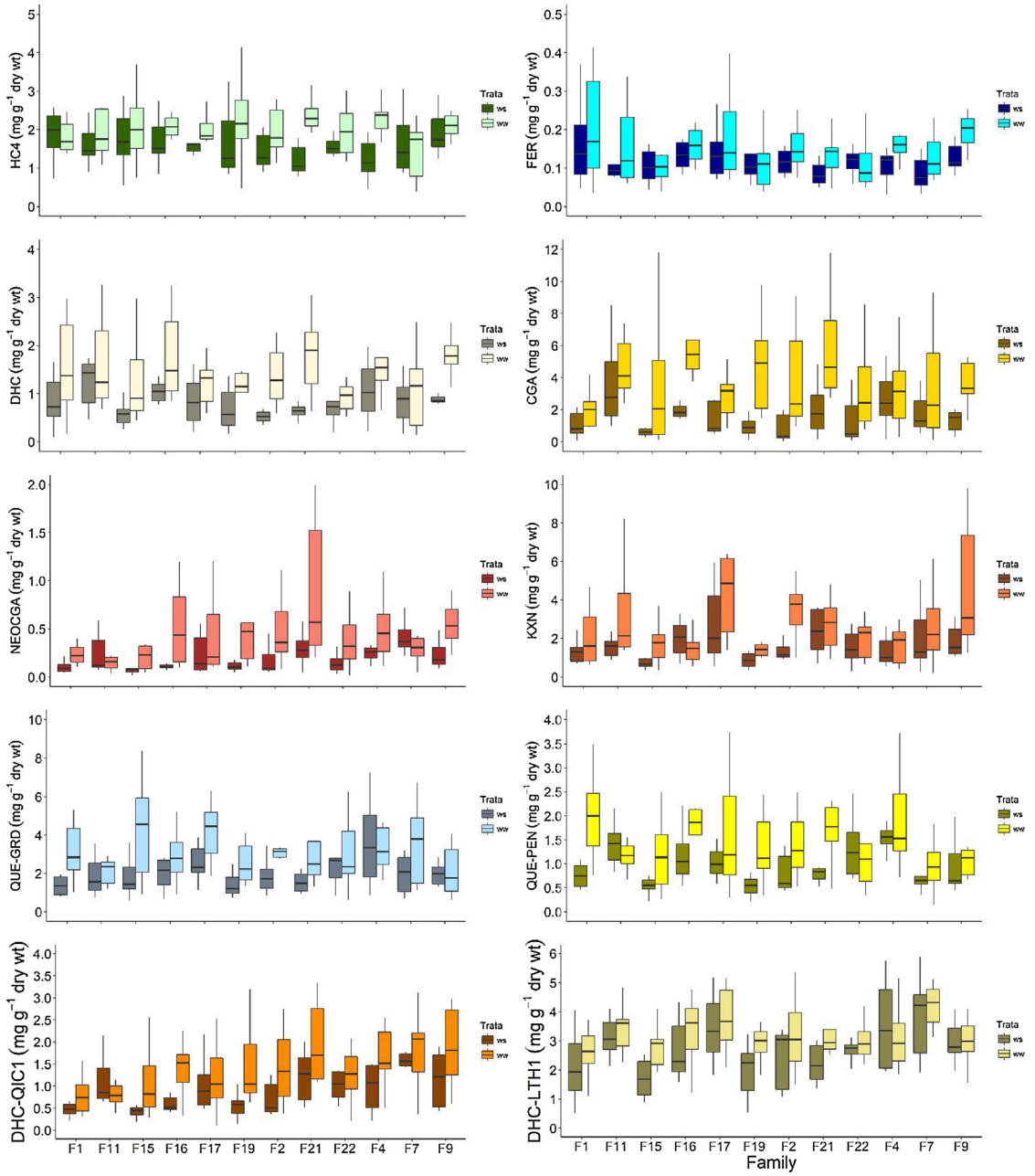
HC4 (mg g⁻¹ dry wt)
FER (mg g⁻¹ dry wt)
DHC (mg g⁻¹ dry wt)
CGA (mg g⁻¹ dry wt)
NEOCGA (mg g⁻¹ dry wt)
KXN (mg g⁻¹ dry wt)
QUE-GRD (mg g⁻¹ dry wt)
QUE-PEN (mg g⁻¹ dry wt)
DHC-QIC1 (mg g⁻¹ dry wt)
DHC-LTH1 (mg g⁻¹ dry wt)
Trata
ws
ww
F1 F11 F15 F16 F17 F19 F2 F21 F22 F4 F7 F9
Family

Fig. 7. Variation in leaf concentration of several phenolic compounds of well-watered (ww – bright colors) and water-stressed (ws – dark colors) leaf seedlings of 11 maternal families coming from the same beech population at the southernmost distribution limit of the species. Compounds associated with phenylpropanoid and flavonoid metabolism. HC4 – p-coumaric acid; FER – ferulic acid; DHC – caffeic acid; CGA – chlorogenic acid; NEOCGA – neochlorogenic acid; KXN – catechin; QUE-GRD – quercetin-*O*-glucuronide; QUE-PEN – quercetin-*O*-pentoside; DHC-QIC1 – caffeoylquinic acid isomer 1; DHC-LTH1 – caffeoyl threonic acid isomer 1.

of the metabolite). There were also a few metabolites with a positive signature in the models, though these had the lowest weight in terms of coefficient value and VIP. Thus, for A_{net}, the saccharides DIS-2184 and SUG-1775 and the terpene phytol were the three compounds with highest positive weighting in the PLS model. In the case of g_{wv}, phytol was almost insignificant, and the disaccharide DIS-2199 joined to the two sugars already assigned to the A_{net} model had the highest positive weighting. The highest negative coefficients in the PLS model for Φ_{PSII} were some of the same as for the A_{net} and g_{wv} models: raffinose, fructose, glucose, ascorbic acid and proline. The response of the amino acid pyroglutamic acid may be distinctive from its role in the other two models, as a metabolite with a negative and significant coefficient in the Φ_{PSII} model. The saccharides SUG-1734 and DIS-2184, phytol and glycolic acid had significant positive relationships with Φ_{PSII}. However, the weight of parameters with positive loading standardized coefficients was higher than those metabolites loading with a negative coefficient.

4. Discussion

4.1. Overall drought response

Understanding the response to water stress by beech is particularly important for the future of the species in the context of climate change. Water stress was the main factor that brought about the most noticeable changes in ecophysiological traits and the metabolome. Diffusional (g_{wv}) and non-diffusional limitations (decrease of Φ_{PSII}) to photosynthesis followed to the imposition of the water stress regardless of

Table 1
Variable importance in the projection value (VIP) and regression coefficients (average ± s.e.) for the PLS-models relating A_{net}, g_{wv} and Φ_{PSII} with the pool of metabolites. Only those metabolites with a significant loading in the model (VIP > 1, confidence interval for coefficients not overlapping) are shown. In cursive are depicted those that were at the limit of significance. The P value represents the CV-ANOVA for the three models, and the quality for the PLS models were signified using the parameters R^2Y and Q^2. Abbreviators of metabolites are described in Table S1 of supplementary material available online.

	A_{net}				g_{wv}				Φ_{PSII}			
PLS-Models	n	R^2	Q^2	P	n	R^2	Q^2	P	n	R^2	Q^2	P
	196	0.743	0.638	***	196	0.744	0.623	***	196	0.635	0.494	***
Metabolite	VIP		regression coefficient		VIP		regression coefficient		VIP		regression coefficient	
RAF	2.85		−0.14 ± 0.03		2.70		−0.13 ± 0.03		2.92		−0.14 ± 0.03	
FRU	2.73		−0.13 ± 0.03		2.47		−0.12 ± 0.04		2.91		−0.14 ± 0.04	
GLC	2.51		−0.12 ± 0.03		2.29		−0.10 ± 0.03		2.68		−0.12 ± 0.03	
ni-1570	1.65		−0.08 ± 0.02		1.72		−0.09 ± 0.02		1.55		−0.07 ± 0.02	
ASC	1.38		−0.08 ± 0.03		1.23		−0.07 ± 0.03		1.67		−0.09 ± 0.03	
GAL	1.81		−0.07 ± 0.01		1.82		−0.07 ± 0.01		1.72		−0.06 ± 0.02	
PRO	1.47		−0.06 ± 0.03		1.40		−0.06 ± 0.02		1.64		−0.08 ± 0.06	
SUG-2024	1.59		−0.06 ± 0.06		1.44		−0.04 ± 0.06		1.55		−0.05 ± 0.04	
SUC	1.49		−0.06 ± 0.03		1.51		−0.06 ± 0.03		n.s.		n.s.	
DIS-2421	1.32		−0.05 ± 0.04		n.s.		n.s.		1.33		−0.05 ± 0.05	
DIS-2546	1.22		−0.04 ± 0.03		n.s.		n.s.		n.s.		n.s.	
VAL	1.44		−0.04 ± 0.02		n.s.		n.s.		1.43		−0.04 ± 0.04	
ASP	1.33		−0.03 ± 0.02		1.35		−0.04 ± 0.02		n.s.		n.s.	
GABA	1.15		−0.03 ± 0.02		1.26		−0.04 ± 0.02		n.s.		n.s.	
ILE	1.35		−0.04 ± 0.02		n.s.		n.s.		1.43		−0.05 ± 0.02	
DIS-2604	1.17		−0.04 ± 0.02		1.21		−0.04 ± 0.02		n.s.		n.s.	
PCA	1.09		−0.05 ± 0.03		n.s.		n.s.		1.43		−0.07 ± 0.05	
DIS-2184	1.19		0.06 ± 0.02		1.37		0.07 ± 0.01		1.16		0.05 ± 0.04	
SUG-1775	*0.93*		*0.05 ± 0.03*		1.04		0.06 ± 0.05		n.s.		n.s.	
PHY	1.14		0.04 ± 0.03				n.s.		1.24		0.05 ± 0.04	
DIS-2199	*0.97*		*0.04 ± 0.03*		1.25		0.06 ± 0.04				n.s.	
GOA	*0.98*		*0.04 ± 0.03*				n.s.		1.19		0.05 ± 0.04	
CBU	n.s.		n.s.		*0.94*		*−0.04 ± 0.03*				n.s.	
DIS-2634	n.s.		n.s.		n.s.		n.s.		1.22		−0.05 ± 0.04	
DIS-2546	1.22		−0.04 ± 0.03		n.s.		n.s.		1.19		−0.04 ± 0.03	
THR	1.21		−0.02 ± 0.01		n.s.		n.s.		1.21		−0.03 ± 0.02	
BET	*0.82*		*0.02 ± 0.02*		n.s.		n.s.		*0.92*		*0.03 ± 0.02*	

maternal family (Rose et al., 2009; Sánchez-Gómez et al., 2013; Aranda et al., 2015). Water stress also caused a similar decrease in Φ_{PSII} among maternal families, but had an inter-family effect on g_{wv} and A_{net}. Although plants were able to maintain net carbon assimilation across the range of the water stresses applied, water stress had a negative effect on growth (see Aranda et al., 2017a for more details). The decoupling of water stress from growth and photosynthesis could be at the root of changes in the balance of carbon allocation to different sinks (Muller et al., 2011), and in particular to the pool of non-structural carbohydrates (NSC) in leaves. Thus, responsiveness to water stress resulted in changes of leaf compounds related to primary and secondary metabolism. From the overall pool of metabolites, those related to carbon and nitrogen pathways were the most affected by water stress. Alterations in the pool of carbohydrates are a frequent an early response to drought, sparking drought tolerance mechanisms (i.e. osmoregulation), and could reflect changes in the internal source-sink carbon demands e.g. for plant growth (Hartmann and Trumbore, 2016; Rivas-Ubach et al., 2016). Paradoxically, although NSCs are primarily photosynthesis-derived metabolites, and net photosynthesis decreased in response to water stress, there was a general increase in concentration of main NSC in leaves (Blessing et al., 2015; Blessing et al., 2016). This probably implies that the decrease of A_{net} was not sufficiently severe to deplete the daily accumulation of carbohydrates in the leaves (Sancho-Knapik et al., 2016), or that interconversion between starch and NSC was enhanced under water stress. Although starch, sucrose, fructose and glucose are submitted to strong seasonal variation in beech (Hoch et al., 2003; El Zein et al., 2011), sucrose and the other three monosaccharides detected were actually highly responsive to water stress (Blessing et al., 2015; Blessing et al., 2016). Thus, the large relative increase in compounds from the main pool of NSC with drought, may be better related to a drop in their use to fuel growth and respiratory demands of WS plants. In fact, carbon demand for leaf respiration in beech has been show to decrease under water stress (Rodriguez-Calcerrada et al., 2010), in a similar way to other species when drought is considered as the only stress factor (Hartmann et al., 2013). Suppression of growth as consequence of water stress in all families, previously shown in Aranda et al. (2017b), kept pace with the increase of the pool of carbohydrates in leaves as previously reported by Peuke et al. (2002) in different beech provenances submitted to drought. Furthermore, the concentrations of metabolites seem to be related to the effective water stress suffered by the plants in terms of predawn leaf water potential (Fig. 1). The entire carbohydrate metabolism comprising some unidentified disaccharides, the pentose ribose and aldopentose arabinose, and the main monosaccharides and sucrose, all responded similarly to water stress. The most noticeable increase resulting from our water stress treatment was that in the bulk leaf concentration of the trisaccharide raffinose. Although its precursor galactinol did not change, both *myo*-inositol a precursor of the galactinol and raffinose an end-product from galactinol, increased considerably in response to water stress (reviewed recently by Sengupta et al., 2015). The non-linear increase in raffinose with Ψ_{pd} suggests that its accumulation is triggered when a water stress threshold is surpassed. Several different functions for raffinose have been suggested, however the physiological role of raffinose in the response to abiotic stresses is yet to be fully elucidated (Sengupta et al., 2015). Two of its biological functions are: to act as an osmo-protectant, a non-enzymatic antioxidant counteracting the accumulation of reactive oxygenic species under stress (reviewed by Elsayed et al., 2014), or as intermediate in the transport and storage of carbohydrates. An alternative explanation could be that the large increase of raffinose in leaves of water-stressed seedlings may be a passive process resulting from that increase in carbon transportation from source to sinks in the plant under water stress. In this respect, raffinose has an important role in sucrose mobilization during active symplastic phloem loading, and these changes in it concentration could be consequence of an increase in phloem transport to enhance the supply of carbohydrates to the belowground organs under moderate drought conditions (Schall

et al., 2012; van Hees, 1997). This process has been reported for seedlings under moderate drought (Hommel et al., 2016) in mixed stands and monocultures of beech using $^{13}CO_2$ labelling. Previously Scartazza et al. (2015) also showed that both a seasonal change and drought response can shift carbon allocation from sources to sinks in beech. It could be hypothesized that lower carbon investment in stem growth in moderately stressed seedlings could explain the accumulation of the pool of carbohydrates despite the drop in the leaf carbon assimilation with water stress (Barthel et al., 2011). Decreased leaf respiration rates and above-ground biomass with water stress in beech (Rodriguez-Calcerrada et al., 2010; Aranda et al., 2017a), both carbon-demanding processes, would reinforce this hypothesis explaining the higher leaf storage of NSC under water stress, and of the raffinose in particular (Körner, 2003; Sala and Hoch, 2009).

The content of malic acid, the most abundant leaf organic acid in plants, together with fumaric and citric acids, increased in response to water stress in most of the maternal families. Leaf concentration of the non-proteinogenic amino acid GABA was also enhanced in response to water stress (Allan et al., 2008; Warren et al., 2011, 2012; Vieira et al., 2017). This change could be related to levels of its precursor glutamate which was also enhanced under water stress. GABA seems to play a role in this plant response to various abiotic stresses, potentially as a direct osmo-protectant, or in water-stress signaling to regulate functional processes such as the stomatal closure (Allan et al., 2008; Gilliham and Tyerma, 2016; Makonnen et al., 2016). Shunting GABA through the TCA cycle into succinic acid could be an important mechanism in nitrogen metabolism, and a means of regulating the C:N balance of plants under stress. Nevertheless, leaf succinic acid content did not change significantly in response to drought in beech, although other up-stream TCA intermediates did (see above). Together the aforementioned changes in major organic acids from the TCA cycle, and similar increases in many amino acids, including the serine family (3-phosphoglycerate derived) e.g. serine or glycine, branched chain amino acids (pyruvate derived) e.g. valine, the aspartate family (oxaloacetate derived) e.g. aspartate, threonine or isoleucine, and the glutamate family (ketoglutarate derived) e.g. glutamate or proline, all related to intermediates of the TCA cycle, were all implicated in the response to water stress. Accumulation of amino acids has previously been described for many plants exposed to abiotic stress, as an indication of cell damage in some species, or considered to have a beneficial effect during stress acclimation in others. In the specific case of isoleucine, its accumulation could be linked to one of its precursors, threonine (Joshi et al., 2010). Thus, an increase in threonine may act as fuel for the downstream increase in isoleucine as a part of a coordinated response to drought resulting in the build up of other branched chain amino acids (BCAAs) e.g. valine. An increased concentration of free amino acids, particularly BCAAs, as osmolytes in response to abiotic stress, is thought to play a role in plant stress tolerance, but this is less commonly reported than, for example, the role of proline in osmo-protection (reviewed in Lehmann et al., 2010), or the role of the aforementioned GABA in stress tolerance (Krasensky and Jonak, 2012). Proline have been described to increase in response to water stress in beech (Schraml and Rennenberg, 2000), and for the extent of this increase to be genetic governed according to population origin (Peuke et al., 2002; Aranda et al., 2017b).

Moderate or intense water stress suppresses growth rather than just photosynthesis, leading to the stimulation of secondary metabolism as carbon sink. The role of secondary metabolites in plant resistance to drought has not been well characterised, and is often hard to explain or predict (Niinemets, 2016). The accumulation of leaf secondary compounds in water-stressed plants has frequently been reported (Ayaz et al., 2000; Griesser et al., 2015; Fernández de Simón et al., 2017), but they can also be unaffected or even diminish under stress, and metabolite-specific responses are common. For instance in a drought-stress experiment with *Eucalyptus* species, and provenances, an increase in secondary metabolites synthetized via shikimate e.g. condensed tannins was reported, and at the same time metabolites from the isoprenoid-synthesis pathway e.g. terpenes were unaffected by drought (McKiernan et al., 2014, 2016). Other secondary compounds and their precursors, in particular those from the phenylpropanoid pathway e.g. shikimic acid or 3-*trans*-caffeoylquinic acid (chlorogenic acid), and those involved in flavonoid biosynthesis e.g. quercetin were down-regulated by water stress in clones of *Eucapyptus globulus* (Correia et al., 2016). The overall decrease that we found in phenolic compounds, both phenyl-propanoids and flavonoids, from beech leaves under water stress was an unexpected result. However, it does agree with that of Correia et al. (2016), who reported lower concentrations of chlorogenic acid and quercetin in the leaves of water-stressed clones of *E. globulus*. The leaf content of several glycosylated derivatives of quercetin and kaempferol significantly decreased in water-stressed plants compared with well-watered plants in our experiment. Water stress also lowered the phenolic acid content of willow clones (Turtola et al., 2005).

Drought has previously been found to increase the tocopherol content of beech leaves (García-Plazaola and Becerril, 2000), and it is assumed to contribute to plant oxidative-stress tolerance (Munné-Bosch, 2005), but this was not the case in our experiment. The shikimate pathway in beech leaves respond to ozone (Betz et al., 2009) and in *Quercus* leaves some compounds e.g. quinic acid are associated with responses to drought, light and temperature stress (Passarinho et al., 2006). However, we did not find significant changes in either shikimic acid or quinic acid, or other related compounds, in response to water stress. These results suggest that the response to water stress in beech that we detected was based to a greater extent on modifications to primary metabolism, than on the reinforcement of photoprotective responses related to secondary metabolism. In fact, there was a decrease in the Φ_{PSII} among all of the maternal families we sampled (Fig. 1), indicating that the potential mechanisms limiting the negative impact of water stress on the leaf photochemistry were ineffective.

4.2. Metabolism and variability among maternal families

Linking plant performance across different biological scales is one of the current challenges in biology. Earlier studies have shown a high intra-specific variability among beech populations in phenotypic traits; ranging from ecophysiological traits (Rose et al., 2009; Fotelli et al., 2009; Sánchez-Gómez et al., 2013; Aranda et al., 2015), to nutrient use (Peuke et al., 2006) or leaf metabolic composition (Peuke et al., 2002; Aranda et al., 2017b). However, less is known about responses at the intra-population level (Aranda et al., 2017b), which may hold one of the main sources of genetic variability within some forest tree species (Ramírez-Valiente et al., 2009), and is fundamental for understanding the potential for adaptation to current and future local conditions (Alberto et al., 2013). Substantial intra-specific genetic diversity in the metabolome has been found among natural populations of beech (Peuke et al., 2002; Aranda et al., 2017b). In the present work, this genetic variability was extended to the intra-population level, specifically open-pollinated families from a marginal population at the south-west edge of the beech distribution area. It seems that intra-population secondary metabolism was more dependent on the genetic background than primary metabolism that followed a more general response pattern to water availability across families (Hochberg et al., 2013). Although there were some general phenotypic responses to water stress shared by all the maternal families we studied, family-specific patterns were evident in the concentration of some specific leaf metabolites, e.g. raffinose differed greatly among families (inset in Fig. 4). The genetic specificity in the metabolic response (covering clonal to provenance variation) has been extensively studied in model forest tree species e.g. *Populus* sp., *Eucalyptus* sp. or *Pinus* sp. (Shvaleva et al., 2006; Dauwe et al., 2012; Barchet et al., 2014; Correia et al., 2016; Fernández de Simón et al., 2017). However, results on non-model tree species such as beech are very scarce. An earlier study described changes in some metabolites e.g. the amino acid proline and some organic acids e.g.

fumaric acid, according to population origin (Aranda et al., 2017b). High genetic variability has previously been observed to govern the response of phenolic compounds to biotic stresses e.g. herbivore pressure (Haviola et al., 2012) or abiotic stresses, e.g. sensitivity to drought (Du et al., 2016). Here we report similar dependence of leaf concentration of specific metabolites according to family for some compounds e.g. proline, chlorogenic acid and secondary metabolites e.g. kaempferol-*O*-*p*-coumaroyl-feruloyl deoxyhexoside under well-watered conditions or in plants submitted to water stress. Despite increasing evidence of the high genetic control in expression of the metabolome, as shown here for beech, it remains a challenge to establish the adaptive value of this apparent genetic control and how it varies according to specific environmental pressures.

4.3. Metabolic and leaf eco-physiological response

Drought reduced net photosynthesis and growth. In the case of net photosynthesis, the decrease was a consequence of the down-regulation of stomatal conductance that increased diffusional limitation to CO_2 uptake, and lowered the quantum yield of PSII. Despite the drought-induced reduction in carbon uptake by beech seedlings (Gallé and Feller, 2007; Sánchez-Gómez et al., 2013; Aranda et al., 2015), the pool of non-structural carbohydrates was not depleted. In fact, the NSC pool increased significantly for all the families grown (Vieira et al., 2017). The interaction of drought and nutrients use is poorly understood in forest tree species, though recently recognized to be important for the interplay between recovery and survival after drought, and the nutritional status of trees (Gessler et al., 2004, 2017). In the specific case of amino acids, there was a noticeable increase in their concentration in leaves of water-stressed seedlings concurrent with an increase of total leaf nitrogen content in beech (reported by Sánchez-Gómez et al., 2013; Aranda et al., 2017a,b), and in other forest tree species under water stress (Aaltonen et al., 2017). The general accumulation of amino acids could be related to an increase in protein degradation (Fotelli et al., 2002), but alternatively to diverting of nitrogen metabolism towards the accumulation of compounds with defensive, antioxidant and osmoprotectant roles. The overall accumulation of a pool of N-rich compounds would be in accordance with the previously observed trend of decrease in the leaf C:N ratio with water stress in beech, as a consequence of the overall increase in the leaf N concentration (Aranda et al., 2017a). This response has also been observed in other species submitted to drought stress (Hochberg et al., 2013). In the context of leaf C:N balance, since the aforementioned changes in carbon metabolism in response to drought warrant attention, these alterations of nitrogen metabolism also deserve greater consideration (Gessler et al., 2017). Though nitrogen supply can increase the drought sensitivity of beech by promoting a proportionally higher investment in aboveground biomass (see Dziedek et al., 2016), the plants' nitrogen metabolism could be much more complex under water stress as supported by our data and a recent review (Gessler et al., 2017).

5. Conclusions

Water stress was the main factor affecting the leaf functional response of beech at the ecophysiological and metabolic levels. Decrease in leaf net photosynthesis, stomatal conductance of water vapour and actual photochemical efficiency of photosystem II (Φ_{PSII}) are well described general ecophysiological responses, which tracked important changes in compounds related to the tricarboxylic acid (TCA) cycle, carbohydrate and amino acid metabolism. Evolution of the pool of carbohydrates could be fundamental to drought tolerance, and consequently, it could contribute to provide light into the renewed interest in the mechanisms of forest trees survival to prolonged water stress (Martínez-Vilalta et al., 2016). Although most metabolites showed a general response to water stress, maternal families (open-pollinated trees) contributed as source of variability in the observed phenotypic changes. This result stresses the relevance of the intra-specific variability in phenotypic traits of beech at different scales of biological organization (Aranda et al., 2017a, 2017b). Metabolic diversity, in particular among secondary metabolites, has adaptive relevance influencing the evolutionary potential of plants (reviewed by Moore et al., 2014). The metabolome can be highly modified according to different genetic backgrounds across scales ranging from populations (Dauwe et al., 2012; Aranda et al., 2017b) to individuals (Barchet et al., 2014; de Miguel et al., 2016; Fernández de Simón et al., 2017). Thus, the intra-population variability in the metabolic and ecophysiological responses we report could be as relevant as the inter-population variability (Frank et al., 2017; Aranda et al., 2017b), and have a high impact in the adaptiveness of beech at the local scale in a context of climate change (Alberto et al., 2013). This capacity relies on the maintenance of enough genetic variability within populations to form the basis for the adaptation to new environmental challenges.

Authors' contribution

I.A., E.C. and B.F.S. designed the experiment; I.A. and D.S.G. measured physiological parameters and analyzed data; I.A. wrote a first draft of the article; EC and BFS analyzed and identified metabolites; I.A., D.S.G., E.C. and B.F.S. discussed results and wrote the final manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.envexpbot.2018.03.014.

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